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Adsorption of *Vibrio parahaemolyticus* onto Chitin and Copepods

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Vibrio parahaemolyticus was observed to adsorb onto chitin particles and copepods. The efficiency of adsorption was found to be dependent on pH and on the concentration of NaCl and other ions found in seawater. Highest efficiency was observed in water samples collected from Chesapeake Bay and lowest in water from the open sea. *V. parahaemolyticus* was found to adsorb onto chitin with the highest efficiency of the several bacterial strains tested. *Escherichia coli* and *Pseudomonas fluorescens* did not adsorb onto chitin. The adsorption effect is considered to be one of the major factors determining the distribution of this species and affecting the annual cycle of *V. parahaemolyticus* in the estuarine system.

The association of microorganisms onto particulate matter is one of the most important phenomena involved in the initiation of biodegradation in the aquatic environment. Many periphytic bacteria, as well as fungi and yeasts, depend on the substratum as a source of organic nutrients and as a site for the concentration of organic substances, which are dissolved in the water (13). In fact, all marine bacteria, fungi, and blue-green algae are potentially periphytic and many are facultative or "part-time" periphytes, being free living under some conditions. Many species are obligate periphytes and depend on a suitable substratum for normal growth (13).

In the case of chitinoclastic bacteria, a particularly important function is that of initiating micro-colonies on chitin in order to degrade, and thereby utilize, chitinous material. In earlier studies of the ecology of *Vibrio parahaemolyticus*, we found that the association of *V. parahaemolyticus* with chitin and copepods was essential for the continuation of the annual cycle of this organism in the Chesapeake Bay (7; T. Kaneko, Ph.D. thesis, Georgetown University, Washington, D.C., 1973). Since *V. parahaemolyticus* produces an active chitinase, the ecological significance of the organism is presumed to involve the recycling of chitinous material, as well as other organic nutrients derived from zooplankton.

The purpose of this study was to elucidate the adsorption of *V. parahaemolyticus*, a chitinoclastic bacterium, on substrata, such as chitin and copepods occurring in situ. Several questions, then, were asked and these concerned the mechanism and function of adsorption, its specificity for a particular organism and/or substratum, differences, if any, in the adsorption of organisms on chitin particles and copepods, and, finally, the role of adsorption in the ecology of microorganisms in the aquatic environment. This paper describes research work done on the adsorption properties of *V. parahaemolyticus* and our conclusions with respect to the objectives stated above.

MATERIALS AND METHODS

Organisms used. Except in the experiments concerning selective adsorption demonstrated by various species, *V. parahaemolyticus* strain SAK 19 was used in the experiments described below. Experiments on selective adsorption were done by using the following: *V. anguillarum* ATCC 14181, *V. marinofulvus* ATCC 14395, *V. marinopraesens* ATCC 14648, *Vibrio* sp. (a Chesapeake Bay strain isolated from a dead soft-shell clam, *Mya arenaria*), *Achromobacter* sp. (a Chesapeake Bay strain isolated from a dead soft-shell clam), *Achromobacter fischeri*, *Proteus vulgaris*, *Citrobacter freundii*, *Escherichia coli*, *Pseudomonas fluorescens*, and *V. parahaemolyticus* SAK 19.

Bacterial cultures used in the experiments were grown for 15 h. For cultures of *Achromobacter* sp., *A. fischeri*, *P. vulgaris*, *C. freundii*, *E. coli*, and *P. fluorescens*, the medium used was a broth composed of proteose peptone (Difco), 1.0%; yeast extract (Difco), 0.3%; NaCl, 0.5%; pH 7.5 ± 0.2. For the other cultures, including *V. parahaemolyticus*, a broth was used composed of proteose peptone (Difco), 1.0%; yeast extract (Difco), 0.3%; NaCl, 2.4%; $MgSO_4 \cdot 7H_2O$, 0.7%; $MgCl_2 \cdot 6H_2O$, 0.53%; KCl, 0.07%; pH 7.5 ± 0.2.

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Copepods and chitin particles used. Copepods used in the experiments were collected from Chesapeake Bay and only adult forms were used. The size of copepods in Chesapeake Bay depends on the individual, as well as the development cycle. The average size range was about 0.5 to 2 or 3 mm. The copepods used were not bacteria-free initially but were washed before the experiments. The number of bacteria associated with copepods depends on the season, with fewer bacteria associated with the copepods in the winter than in the summer. Approximately 10^5 to 10^6 bacteria were found per gram of plankton (wet weight) in the winter months. Chitin particles were obtained by grinding commerical chitin (Sigma Chemical Co.) and sieving the ground chitin so that chitin particles used were in a size range of 250 to 500 μm.

Basic procedure followed in the adsorption tests. Forty milliliters of about 0.5 g of chitin (dry weight) or copepods (wet weight) suspension was added to 150-ml flasks. The chitin particles and copepods were prewashed with appropriate solutions to remove the very fine particles filterable through a plankton net (8 by 8 cm, 77-μm opening). Bacterial suspensions were added to the chitin or copepod suspensions after the bacterial cultures had been washed with the same solution as that used to make up the chitin or copepod suspensions. The mixtures were then well mixed by gentle agitation. The final concentration of bacteria was about 10^7/ml. A portion of the mixture (2 ml) was taken up in Pasteur pipettes and filtered through a presterilized plankton net to remove the chitin particles or copepods. The filtrate was used in the determination of the bacterial counts, providing the number of bacteria in the free state at zero time. The bacterial counts were determined by the most probable number method. The bacteria-chitin (or copepod) mixture was then placed on a shaker at a constant speed at 20 C for the remainder of the given experiment. Except where stated otherwise, the mixture was shaken for 6 h. The time period used was determined in advance, i.e., the time period selected was that which was short enough so that growth did not occur and of such a duration that die-off did not occur. Bacterial counts were determined several times during the 6-h period on the shaker. Controls, i.e., bacterial solutions without chitin or copepods added, were also run in order to detect any experimental errors which might influence the adsorption rate calculations.

Adsorption of V. parahaemolyticus onto chitin particles in natural estuarine water. Rhode River water, aged for 4 months (4.2‰ salinity, pH adjusted to 7.0), was used in experiments carried out to determine the adsorption of *V. parahaemolyticus* strain SAK 19 onto chitin particles. The Rhode River water was sterilized by a membrane filter (Millipore Corp.) (0.45 μm). Chitin particles and organisms were washed with the same sterile water, as described above for the salts solutions. Bacterial counts were determined, after filtration of the chitin particles, at 0, 2, 4, and 6 h. Controls were also run in all of these experiments.

Adsorption of V. parahaemolyticus onto chitin particles in natural estuarine water of various salinities. Similar experiments as those described above were carried out using water of different salinities collected at several locations in Chesapeake Bay during a cruise of the R/V Ridgely Warfield (Johns Hopkins University Research Vessel) in May 1972. Salinities of each of the estuarine water samples used were 1.6, 4.0, 8.9, 9.7, 12.0, 14.5, and 15.8‰, respectively, and the pH of each sample was 8.0, 8.2, 8.6, 8.4, 8.9, 8.9, and 8.0, respectively.

Adsorption of V. parahaemolyticus onto chitin and copepods in natural seawater. To detect any differences in adsorption of organisms onto chitin and copepods arising from ionic composition, NaCl solutions and natural seawater were compared. That is, a comparison of adsorption occurring in diluted seawater, full strength seawater, and in different concentrations of NaCl was done. The natural seawater was collected from off the Georgia coast in the summer of 1971, the salinity of which was 35.3‰. The pH of the water samples used in this set of experiments was adjusted to 7.0.

Effect of pH on adsorption. To determine the effect of pH on the adsorption of *V. parahaemolyticus* onto chitin particles, experiments were done using phosphate-buffered 1.0% NaCl solutions in the pH range 4.3 to 10.5. At the extreme acid and alkaline pH, the number of bacteria was adjusted by reference to controls run with the test cultures.

Effect on mono- and divalent ions on adsorption. Salts, such as $MgSO_4$, $MgCl_2$, $CaCl_2$, and KCl, were used to determine the effect of these ions on the adsorption of *V. parahaemolyticus* onto chitin particles. All experiments were run using 1.0% NaCl solutions (pH 7.0) with 0, 0.01, 0.1, and 1.0% of each ion added to the solution. The concentration of each salt was selected with reference to Herbst's artificial seawater (3), consisting of NaCl, 3.0%; KCl, 0.07%; $MgCl_2 \cdot 6H_2O$, 1.08%; $MgSO_4 \cdot 7H_2O$, 0.54%, and $CaCl_2 \cdot 2H_2O$, 0.1%.

Selective adsorption of organisms occurring among different bacteria species. To determine whether the adsorption effect is specific to *V. parahaemolyticus*, several bacterial strains representing several genera (listed above) were used. Two different kinds of water were used, a 1.0% NaCl solution (pH 7.0), and Rhode River water (4.2‰ salinity, pH 7.0). Bacterial counts were carried out at 0 and 6 h, respectively.

RESULTS

Adsorption of V. parahaemolyticus onto chitin and copepods. Results of the adsorption experiments using *V. parahaemolyticus* and chitin in natural estuarine water (4.2‰ salinity) are shown in Fig. 1. Bacteria were found to adsorb onto the chitin particles (250- to 500 μm particles) and a linear association with time was noted. The initial bacterial counts, about 10^7 to 10^8, i.e., of the free cells in the chitin-estuarine water mixture, dropped to 10^4 to 10^5 within 6 h. The number of bacteria in the controls without chitin did not change in the 6-h period. Results

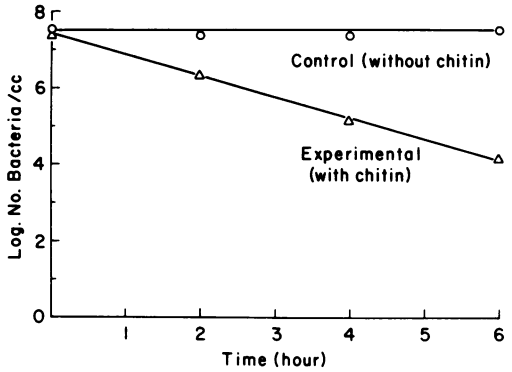

FIG. 1. *Adsorption of V. parahaemolyticus onto chitin in natural estuarine water. Rhode River water (4.2‰ salinity) was used. pH was adjusted to 7.0.*

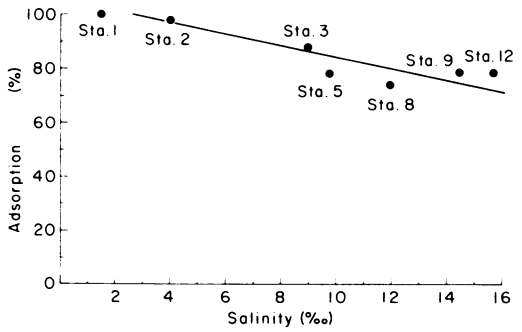

FIG. 2. *Adsorption of V. parahaemolyticus onto chitin in natural estuarine water collected at various stations during the R/V Ridgely Warfield cruise, May 1972.*

using estuarine water samples collected from various stations in Chesapeake Bay in the same experiment showed clearly that the efficiency of adsorption is related to the salinity of the water (see Fig. 2). Namely, water collected in the Upper Bay, i.e., water of 1.6‰, at Station 1, yielded nearly 100% adsorption. Water (10 to 16‰ salinities) collected between Stations 5 and 12 in the Lower Bay yielded 70 to 80% adsorption. Thus, adsorption efficiency is greater in water of lower salinity.

When NaCl solutions of various concentrations were used, greater adsorption than in estuarine water was noted (Fig. 3). Adsorption in the NaCl solutions was nearly 100% at salinities up to 17‰ and decreased linearly at NaCl concentrations above 17‰. About 70% of the cells were adsorbed at 30‰ salinity. Where diluted and undiluted natural seawater collected from off the Georgia coast was used, the salinity of which was 35.3‰, the efficiency of adsorption was found to be lower than for the NaCl solutions. For example, 100% adsorption was observed at 4‰ salinity, but decreased to about 60% linearly at 30‰ salinity.

When copepods were used instead of chitin particles, less adsorption was noted than for chitin (Fig. 3). About 70% of the bacteria were adsorbed onto copepods at 5‰ salinity, in the case of the NaCl solutions, and 50% adsorption was observed at the same salinity, in the case of the natural seawater. However, adsorption dropped rapidly to 0% at 15‰ for seawater and 22% at the same salinity for the NaCl solution. Thus, there was a difference in adsorption for chitin particles and copepods.

Effect of pH on adsorption. Figure 4 shows the adsorption of *V. parahaemolyticus* onto chitin in 1.0 % NaCl solutions with the pH adjusted as indicated. The NaCl solution used

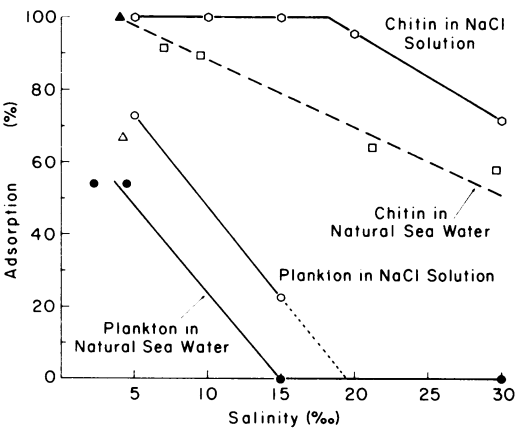

FIG. 3. *Adsorption of V. parahaemolyticus onto chitin and plankton in NaCl solution and seawater at different salinities. (▲) indicates adsorption with chitin in Rhode River water, (△) indicates adsorption with plankton in Rhode River water.*

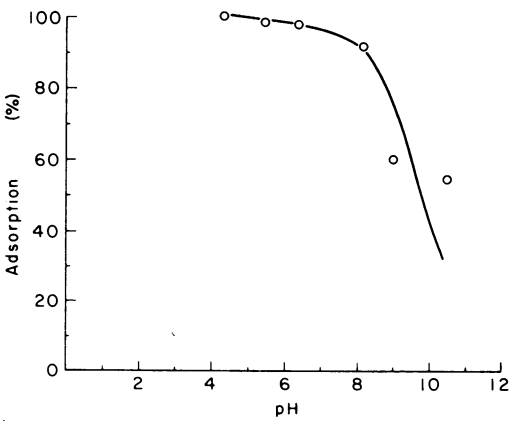

FIG. 4. *Adsorption rate of V. parahaemolyticus onto chitin in 1% NaCl solutions of varying pH.*

was selected to maintain viability since *V. parahaemolyticus* requires NaCl. Adsorption was about 100% at pH 4 and 5 and >90% adsorption was observed at pH 5.0 to 8.0. Increased pH (>8.0) resulted in rapidly decreased adsorption, with 60% at pH 9.0 and 50% at pH 10.0, respectively. Thus, pH strongly affected adsorption.

Since the pH of Chesapeake Bay surface water is between 7.0 and 9.0, and the pH of bottom water is lower than that of surface water (5), adsorption occurring in the natural estuarine water must depend on the pH of the water at any given location, as well as on salinity.

Effect of mono- and divalent ions on adsorption. As shown in Fig. 5, the results of the experiments using $MgSO_4$ and $MgCl_2$ revealed that Mg^{2+} influenced adsorption at concentrations of these ions usually found in seawater (3). As seen in the case of the influence of the other ions on adsorption, the efficiency of adsorption was found to be dependent upon the given ion, as well as ionic strength.

Selective adsorption. A species selectivity for adsorption was observed (Table 1). In 1.0% NaCl, *V. anguillarum* and *V. parahaemolyticus* adsorbed with highest efficiency. The *Achromobacter* and *Vibrio* spp. isolated from dead soft-shell clams, showed relatively high adsorption. *A. fischeri* and *P. vulgaris* adsorbed only very slightly. Adsorption of *C. freundii* and *E. coli* was negligible. When natural estuarine water (4.2‰ salinity) was used, *V. marinofulvus* showed highest adsorption, with *V. parahaemolyticus* next highest. Strains of *E. coli* and *P. fluorescens* isolated from the Chesapeake Bay showed no adsorption. Thus it is clear that adsorption is strain dependent.

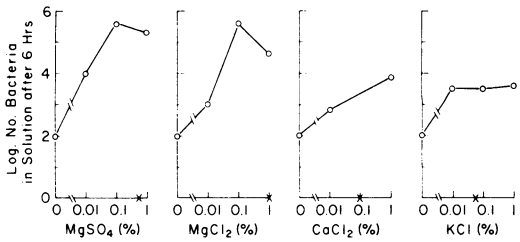

FIG. 5. *Effect of mono- and divalent ions on adsorption of V. parahaemolyticus onto chitin particles in 1% NaCl solution. (×) indicates the concentration of each ion in artificial seawater (Herbst's (3), see Materials and Methods). pH was adjusted to 7.0.*

TABLE 1. *Species specificity of the characteristic of adsorption onto chitin*

Organism	No. of bacteria in solution in the free state	
	Zero time	After 6 h
1.0% NaCl solution		
Vibrio anguillarum ATCC 14181	10^{7}[a]	10^{3}
V. parahaemolyticus SAK 19	10^{7}	10^{3}
Achromobacter sp.[b]	10^{7}	10^{4}
Vibrio sp.[b]	10^{7}	10^{5}
A. fischeri	10^{7}	10^{6}
Proteus vulgaris	10^{7}	10^{6}
Citrobacter freundii	10^{7}	10^{7}
Escherichia coli	10^{7}	10^{7}
Rhode River water (4.2‰ salinity)		
V. marinofulvus ATCC 14395	10^{7}	10^{3}
V. parahaemolyticus SAK 19	10^{7}	10^{4}
V. marinopraesens ATCC 19648	10^{7}	10^{6}
E. coli	10^{7}	10^{7}
Pseudomonas fluorescens[b]	10^{7}	10^{7}

[a] Number of bacteria expressed per milliliter of solution.

[b] Strains isolated from Chesapeake Bay.

DISCUSSION

Adsorption is important in association or aggregation occurring amongst microorganisms, as well as organic matter in the water column, with substrata, i.e., those structures with which the organisms in question maintain, temporarily or permanently, a close contact. In this study, the phenomenon of *V. parahaemolyticus* adsorption onto chitin and copepods was observed. Since *V. parahaemolyticus* is a chitinoclastic organism, its association with copepods is considered to be one of the most important relationships determining its natural habitat as well as its ecological niche (T. Kaneko, Ph.D. thesis, 1973). Ecological surveys of *V. parahaemolyticus* in Chesapeake Bay (7; T. Kaneko, Ph.D. thesis, 1973) and in the Atlantic Ocean (8) led to the conclusion that this organism is an estuarine organism, with its distribution markedly restricted to the estuarine environment. *V. parahaemolyticus* was not isolated from water and sediment samples collected off the South Carolina and Georgia coasts, even from those samples collected only 4 to 5 miles from shore in the summer months (8). Since the organism grows well in seawater-based media, suggesting a slight halophilism, an interesting question arises, i.e., why is the distribution of this organism limited to the estuary? If the association of *V. parahaemolyticus* with chitin or chitin-bearing copepods is considered necessary from the point of view of the annual cycle of this organism in nature (7), then the adsorptive properties of this organism take on great ecolog-

ical significance and it becomes important to discuss those factors affecting adsorption.

Adsorption was observed to occur when chitin particles were added to suspensions of *V. parahaemolyticus* prepared with natural estuarine water (4.2‰ salinity, see Fig. 1). Obviously, most of the bacteria were very quickly adsorbed onto the chitin. Adsorption effect was found to be influenced drastically by salinity. If natural estuarine water of various salinities, collected at several stations in Chesapeake Bay, was used, adsorption decreased with increase in salinity (see Fig. 2). Almost 100% adsorption occurred at a salinity of about 4‰, whereas 70 to 80% was observed at 10 to 16‰. This observation is very important in terms of the distribution of this organism.

pH was also found to affect adsorption significantly (see Fig. 4). An acidic pH was more favorable for adsorption than an alkaline pH. At about pH 8.0 to 9.0, a decrease in adsorption was noted. The pH of natural seawater is about 8.0 to 8.5 and would, therefore, affect adsorption.

Effect of the various ionic components of seawater, other than NaCl, on adsorption was also observed (see Fig. 5). Divalent cations, such as Mg^{2+}, strongly inhibited adsorption. Monovalent ions, such as K^+, produced a milder inhibition than Mg^{2+}. Thus, the concentration of these ionic constituents of seawater, including Na^+, Cl^-, affected adsorption, with a decrease with increased concentration of these ions. This observation was corroborated when adsorption in seawater was compared with that in NaCl solutions (see Fig. 3), although this observation was the opposite of that of Marshall et al. who used a marine *Pseudomonas* sp. (10) for their studies of the reversibility of adsorption.

The mechanism of adsorption of *V. parahaemolyticus* onto chitin or copepods is not completely clear, although some explanation can be provided. If the chitin particles, which were not chemically pure, are positively charged, the association of bacteria, which are negatively charged, with the particles can be explained in rather simple terms. The inhibition of adsorption by the various ions tested, and the positive/negative attraction would be a function of electrokinetic potential (9), i.e., the difference in potential between the medium and the surface of the particle is influenced by salt concentration and pH (6). In the case of copepods, the surface, i.e., the exoskeleton, is, like other crustaceans, covered by a wax layer secreted by the tegmental gland (4). Materials secreted by the plankton may collect on the surface of plankton (12) complicating further the electrokinetics of the copepod surface. Therefore, the net charge on the surface of copepods can be modified and, possibly, may be only weakly positive, although which substances act to modify the surface charge is not known.

This assumption is substantiated by the fact that adsorption of bacteria onto copepods was less than that on chitin particles (see Fig. 3).

The bacterial surface is also modified by various substances produced by the bacteria themselves and the net charge of the bacterial surface depends on the species (2). Species specificity of the adsorption characteristic observed by Marshall et al. (11) was also observed in this study (Table 1). This proved to be interesting, in regard to the ecological role of bacteria. *V. parahaemolyticus* is one of the several bacterial species found to adsorb with highest efficiency. This observation is very important if this bacterium must compete for an ecological niche. That is, this characteristic is of special importance if the organism is a pioneer (1), i.e., the first to attack a substrate and proliferate in competition with other species. In this study, a clear difference in the capacity for adsorption was noted, as for example between *V. parahaemolyticus* and *E. coli*.

The capability to degrade chitin was not, however, found to be directly correlated with adsorption. However, in the case of *V. parahaemolyticus* the high efficiency of adsorption is favorable to the species in that its attachment to chitinous materials provides a nutrient source which it can utilize. From the ecological view, if adsorption occurs, it is better if the organism is not easily separated from its substratum, in order that it be able to multiply on the surface of the substratum. In this study, the association or attachment brought about via initial adsorption, turned out indeed to be stable. A strong attachment between bacteria and substratum, the chitin particle in this case, was observed (T. Kaneko and R. Colwell, unpublished data). Since some bacteria produce gum-like compounds, such as capsules, if the bacteria attach via electrostatic force, those substances may favor prolonged stable attachment (2).

It is concluded from the results of this study that the distribution of *V. parahaemolyticus* is restricted to estuaries, such as the Chesapeake Bay because of the effects of salinity, temperature, pH, and other, as yet unidentified, factors on the attachment, survival, and growth of this organism. The Rhode River in Chesapeak Bay,

where an earlier study of the ecology of *V. parahaemolyticus* was extensively investigated (7; T. Kaneko, Ph.D. thesis, 1973), thus has proven to be an ideal site for the organism to continue its annual cycle.

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LITERATURE CITED

1. Alexander, M. 1972. Microbial ecology. John Wiley and Sons, Inc. New York.
2. Corpe, W. A. 1974. Periphytic marine bacteria and the formation of microbial film on solid surfaces. *In*, R. R. Colwell and R. Y. Morita (ed.), Effect of the ocean environment on microbial activity. University Park Press, Baltimore.
3. Galtstoff, P. S., F. E. Lutz, P. S. Welch, and J. G. Needham. 1937. Culture methods for invertebrate animals. Comstock Publishing Co. Inc., New York.
4. Hickman, C. P. 1967. Biology of the invertebrates. C. V. Mosby Co. St. Louis, Mo.
5. Hires, R. I., E. D. Stroup, and R. C. Seitz. 1963. Atlas of the distribution of dissolved oxygen and pH in Chesapeake Bay 1949-1961. Graphical Summary Report 3. Chesapeake Bay Institute. The Johns Hopkins University, Baltimore.
6. James, A. M. 1957. The electrochemistry of the bacterial surface. Prog. Biophys. Chem. **8**:95-142.
7. Kaneko, T., and R. R. Colwell. 1973. Ecology of *Vibrio parahaemolyticus* in Chesapeake Bay. J. Bacteriol. **113**:24-32.
8. Kaneko, T., and R. R. Colwell. 1974. Distribution of *Vibrio parahaemolyticus* and related organisms in the Atlantic Ocean off South Carolina and Georgia. Appl. Microbiol. **28**:1009-1017.
9. Lamanna, C., and M. F. Mallette. 1965. Basic bacteriology—its biological and chemical background, 3rd ed. The Williams and Wilkins Co. Baltimore.
10. Marshall, K. C., R. Stout, and R. Mitchell. 1971. Mechanism of the initial events in the sorption of marine bacteria to surfaces. J. Gen. Microbiol. **68**:337-348.
11. Marshall, K. C., R. Stout, and R. Mitchell. 1971. Selective sorption of bacteria from seawater. Can. J. Microbiol. **17**:1413-1416.
12. ZoBell, C. E. 1943. The effect of solid surfaces upon bacterial activity. J. Bacteriol. **46**:39-56.
13. ZoBell, C. E. 1972. Substratum-bacteria, fungi and blue green algae. *In* O. Kinne (ed.), Marine ecology, vol. 1, p. 1251-1270. Wiley-Interscience, New York.